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Anti-estrogens such as tamoxifen are important therapeutic agents in the treatment and chemoprevention of breast cancers. Other compounds such as phytoestrogens, fatty acid amides and retinold X receptor (RXR) agonists are also effective against breast cancer in cell lines and in animal models. Because these compounds are unrelated, it has not been appreciated that they might act through a common mechanism. These compounds all share the ability to activate a heterodimer of the steroid and xenobiotic receptor (SXR) and RXR. Our hypothesis is that SXR serves as a common molecular target for some of the anti-proliferative effects of these compounds and that activation of SXR is itself anti-proliferative. We have analyzed mRNA from breast cancer cell lines treated with SXR activators by micorarray, and have validated some of the putative SXR target genes found using quantitative real time RT-PCR. We have also analyzed the mechanism by which SXR activation stops cell proliferation using assays for apoptosis and necrosis. Our preliminary results indicate that SXR activators may be inducing caspase-dependent apoptosis to stop the apparent cell proliferation. Further experiments are underway to study SXR activator-induced apoptosis and link this effect back to the induced SXR target genes found by microarray.

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INTRODUCTION

Anti-estrogens such as tamoxifen are important therapeutic agents in the treatment and chemoprevention of breast cancers. Other compounds such as phytoestrogens, fatty acid amides such as anandamide and retinoid X receptor (RXR) agonists are also effective against breast cancer in cell lines and in animal models. Because these compounds are unrelated, it has not been appreciated that they might act through a common mechanism. These compounds all share the ability to activate a heterodimer of the steroid and xenobiotic receptor (SXR) and RXR. Our hypothesis is that SXR serves as a common molecular target for some of the anti-proliferative effects of these compounds and that activation of SXR is itself anti-proliferative. To this end, we have found that a constitutively active form of SXR, VP16-SXR is able to slow the growth of transiently transfected breast cancer cells similar to treatment with SXR activators. We have detected the expression of SXR in ductal carcinomas, but not in normal breast tissue raising the possibility that the presence or absence of SXR may be related to breast cancer treatment outcome. We have analyzed mRNA from breast cancer cell lines treated with SXR activators by micorarray, and have validated some of the putative SXR target genes found using quantitative real time RT-PCR. We have also begun to analyze the mechanism by which SXR activation stops cell proliferation using assays for apoptosis and necrosis. Our preliminary results indicate that SXR activators may be inducing caspase-dependent apoptosis to stop the apparent cell proliferation. Further experiments are underway to study SXR activator-induced apoptosis and link this effect back to the induced SXR target genes found by microarray.

BODY

Task 1. To determine whether the anti-proliferative effects of phytoestrogens, fatty amides, anti-estrogens and RXR agonists are due to activation of SXR/RXR in breast cancer cells by these compounds. (months 1-24)

Test whether the inhibition of SXR activation with ET-743 blocks the anti-proliferative effects of SXR activators.

We applied to obtain the SXR antagonist ET-743 from its commercial manufacturer PharmaMar, and to date, we have been unsuccessful obtaining this compound to test. PharmaMar is currently not filling requests for this compound as it is in clinical trials. Interestingly, research in our other

projects has identified certain polychlorinated biphenyls (PCBs) as novel SXR antagonists. Notably, we found that several non-planar PCBs could act as potent SXR antagonists (Tabb et al., 2004). The most potent of these, PCB197, has a K_i of 0.6 μM, which establishes it as a reasonably potent antagonist. MCF-7 cells were treated with different concentrations of the SXR activator rifampicin (RIF) in the presence or absence of increasing amounts of the SXR antagonist PCB197 for 7 days, and then cell proliferation was measured using the CyQuant cell proliferation assay (Molecular Probes). Unfortunately, PCB197 had some growth inhibitory effect on MCF-7 cells on its own (see Figure 1; 197 1, 197 5 and 197 10 compared to DMSO control).

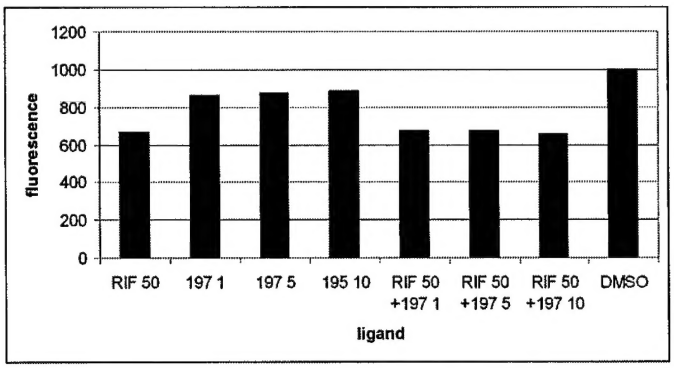

Figure 1. Cell proliferation of MCF-7 cells treated with 50 uM rifampicin (RIF 50) in the presence of increasing amounts of the SXR antagonist PCB197, 1 uM PCB197 (197 1), 5 uM PCB197 (197 5) or 10 uM PCB197 (197 10). Relative fluorescence is a measure of total cell number.

PCB197 was also not able to restore cell proliferation in the presence of 50 μM RIF to the levels of the DMSO control. We will attempt this experiment again using different concentrations of RIF and PCB197 as well as other SXR activators that have previously demonstrated an effect on cell proliferation such as the anti-fungal clotrimazole and the fatty amide anandamide.

Task 2. Define the mechanism by which SXR activation inhibits the proliferation of breast cancer cells (months 12-36).

Microarray analysis of mRNAs from breast cancer cell lines treated with SXR activators (months 12-24).

In order to determine what SXR target genes were responsible for the anti-proliferative effect seen in breast cancer cells after treatment with SXR activators, we initiated a two-pronged approach with two different microarray strategies. The first strategy employed the use of mRNA isolated from MCF-7 ER+ cells treated with three different classes of SXR activators. The SXR activators used were the endogenous cannabinoid anandamide, the anti-estrogen tamoxifen and the macrolide antibiotic rifampicin. RNA from treated cells was used to probe the Affymetrix

U95 Human Microarray. In this approach, we were interested in the genes that could either be up-regulated or down-regulated by all three SXR activators. We anticipated that each compound would produce its own unique signature of regulated genes, but those regulated similarly by all of the compounds should be bona-fide SXR target genes.

Using this first method, we found a total of 21 genes were up-regulated by the three SXR activators (Table 1), and 34 genes were down-regulated (Table 2).

Table 1. Genes up-regulated by three different SXR activators in MCF-7 cells. Values shown are fold increase over control.

Anandamide	Tamoxifen	Rifampicin	gene
2.6	2.8	3.2	**H.sapiens pilot mRNA**
2.7	6.5	5.0	**Human HRY gene**
2.6	2.0	3.4	**Human insulin-like growth factor binding protein-4 (IGFBP4)**
3.6	7.9	4.6	**Homo sapiens mRNA for TGF-beta superfamily protein**
5.2	3.4	6.3	**H.sapiens mRNA for B2-bradykinin receptor**
1.8	2.1	1.9	Homo sapiens mRNA for KIAA0991 protein
1.8	2.2	1.9	Homo sapiens clone 24772 BDP-1 protein mRNA
1.9	2.4	2.0	**Human estrogen-responsive B box protein (EBBP) mRNA**
2.2	7.8	6.3	**Human TR3 orphan receptor mRNA**
2.4	2.6	2.0	**Human calmodulin (CALM1) gene**
2.2	2.0	1.7	**Human AH-receptor mRNA**
3.1	4.0	2.3	Human c-jun proto oncogene (JUN),clone hCJ-1
1.6	2.4	2.6	Neurofibromatosis 2 Tumor Suppressor
2.2	1.9	2.1	Human membrane glycoprotein 4F2 antigen heavy chain mRNA
1.7	5.2	1.9	Human testicular inhibin beta-B-subunit mRNA
2.9	2.7	2.3	Human transferrin receptor mRNA
2.0	2.4	2.3	Human T-cell receptor Ti rearranged gamma-chain mRNA V-J-C region
3.1	2.4	3.8	Homo sapiens A28-RGS14p mRNA
2.2	2.7	2.0	Homo sapiens IPL (IPL)
2.1	1.9	1.6	Human E16 mRNA
1.6	2.4	1.9	Human c-myc-P64 mRNA

Table 2. Genes down-regulated by three different SXR activators in MCF-7 cells. Values shown are fold decrease compared to control.

Anandamide	Tamoxifen	Rifampicin	gene
-7.1	-2.5	-5.6	Human tumor antigen (L6) mRNA
-1.9	-2.1	-1.5	Human 14-3-3 epsilon mRNA
-3.7	-2.1	-1.5	H.sapiens mRNA for nuclear protein SA-2
-1.9	-1.0	-2.3	Human cellular oncogene c-fos
-3.1	1.3	-2.6	Human cyclin G2 mRNA
-2.6	-1.5	-3.7	Human epidermal growth factor receptor (HER3) mRNA
-2.1	-2.4	-2.6	p50-NF-kappa B homolog
-2.8	-2.9	-3.1	Homo sapiens E1B 19K/Bcl-2-binding protein Nip3 mRNA

-1.8	-5.1	-2.3	Insulin receptor substrate-1
-1.5	-1.6	-1.5	H.sapiens tpr mRNA
-2.5	-2.2	-3.8	Homo sapiens mRNA for KIAA0677 protein
-2.0	-3.9	-5.5	Human MXI1 mRNA
-2.2	-1.6	-1.8	Homo sapiens immunoglobulin lambda gene locus DNA
-2.3	-1.8	-3.1	Cluster Incl. AI148772:qc69h01.x1 Homo sapiens cDNA
-2.2	-3.1	-2.7	Human DNA-dependent protein kinase catalytic subunit
-1.5	-1.8	-2.1	Cluster Incl. AI056696:oz26h05.x1 Homo sapiens cDNA
-3.7	-3.0	-3.8	Human extracellular protein (S1-5) mRNA
-4.7	-3.7	-4.0	Cluster Incl. AI445461:tj34g07.x1 Homo sapiens cDNA
-2.1	-1.9	-2.8	Homo sapiens mRNA for KIAA0480 protein
-3.5	-2.3	-3.4	Cluster Incl. AL049250:Homo sapiens mRNA
-1.9	-2.2	-1.9	Human mRNA for KIAA0067
-1.4	-1.5	-1.4	Cluster Incl. AI375033:ta66e10.x1 Homo sapiens cDNA
-3.4	-3.3	-3.5	Homo sapiens mRNA for dia-156 protein
-3.0	-2.8	-3.5	Homo sapiens GBAS (GBAS) mRNA
-1.9	-2.5	-2.1	Cluster Incl. AA845349:ak01g01.s1 Homo sapiens cDNA
-2.7	-1.6	-3.2	Human novel protein AHNAK mRNA
-1.7	-3.0	-2.3	Human beta 3-endonexin mRNA, long form and short form
-1.9	-2.0	-2.4	Cluster Incl. AI659108:tu08c09.x1 Homo sapiens cDNA
-2.3	-1.9	-2.5	Cluster Incl. X87613:Human mRNA skeletal muscle abundant protein
-1.7	-2.2	-1.4	Human protein serine/threonine kinase stk1 mRNA
-3.4	-2.2	-1.7	H.sapiens AUH mRNA
-3.0	-5.6	-1.7	Cluster Incl. W28214:45f7 Homo sapiens cDNA
-1.3	-1.5	-1.5	Cluster Incl. T89651:yd99a05.s1 Homo sapiens cDNA

The second microarray strategy we employed to discover SXR target genes in breast cancer cells utilized the ER+ cell line MCF-7 and the ER- cell line MDAMB231. Both cell types were treated with the potent SXR activator rifampicin, RNA was isolated and then used to probe the Affymetrix U133 Human Microarray. In this approach, we were interested in the genes that could be regulated similarly by SXR activator in both estrogen-receptor positive and negative cell lines. We had previously shown that both types of cell lines demonstrated a reduced proliferation in the presence of SXR activators like rifampicin. Once again, we anticipated that each cell type would produce its own unique signature of regulated genes, but those regulated similarly by rifampicin in both cell types should be bona-fide SXR target genes.

Using this second method, we found many genes either up or down-regulated by rifampicin in either MCF-7 or MDAMB231 cells (Tables 3, 4, 5, 6). By comparing the up and down-regulated lists, we found only 8 genes were up-regulated by rifampicin in both MCF-7 and MDAMB231 cells (Table 7). No genes were found that were commonly down-regulated by rifampicin treatment in both cell lines.

Table 3. Genes up-regulated in U133 Array in MCF-7 cells treated with rifampicin.

Gene description	***fold increase***
JuSo MUC18 glycoprotein mRNA, melanoma adhesion molecule	12.1
Interleukin 7 (IL7)	12.1
Coronin, actin-binding protein, 2A (CORO2A)	9.8
Consensus includes gb:AK025072	6.5
Human hairy and enhancer of split (Drosophila) homolog 2 (HES2)	4.6
G protein-coupled receptor 32 (GPR32)	3.7
Homo sapiens cDNA similar to SEGREGATION DISTORTER PROTEIN	2.5
Histamine H4 receptor (HRH4)	2.5
Homo sapiens hypothetical protein FLJ13962 (FLJ13962)	2.5
Consensus includes gb:AL080106.1	2.3
Protease, serine, 7 (enterokinase) (PRSS7)	2.3
Consensus includes gb:AW592563	2.1
Consensus includes gb:AK022885	2.0
spinocerebellar ataxia (forms 1, 2, 7)	2.0
SWISNF related, matrix associated (SMARCA1)	1.9
Distal-less homeobox 4 (DLX4)	1.7
Lipase, endothelial (LIPG)	1.7
2,3-bisphosphoglycerate mutase (BPGM)	1.8
Calreticulin	1.6
Ephrin receptor EPHA3 secreted form (EPHA3)	1.6
UDP-N-acetyl-alpha-D-galactosamine	1.6
Consensus includes gb:AL567227	1.6
Thrombospondin-1 gene	1.6
Solute carrier family 21 (organic anion transporter)	1.6
gb:BC004917.1 /DEF=Homo sapiens, clone MGC:4771	1.6
Nuclear receptor subfamily 4A2 (NR4A2, Nurr1)	1.6
HLA class II region expressed gene KE4 (HKE4)	1.5
Similar to somatostatin receptor 2	1.5
Emopamil-binding protein (sterol isomerase)	1.5
Similar to chaperonin containing TCP1, subunit 8 (theta)	1.5
MLL-AF4 der(11) fusion protein mRNA	1.5
Protocadherin 17 (PCDH17)	1.5
Consensus includes gb:AW451711	1.5
Consensus includes gb:AW970584	1.5
Oxytocin receptor (OXTR)	1.5
Carbonic anhydrase IV (CA4)	1.5
2,3-cyclic nucleotide 3 phosphodiesterase (CNP)	1.5
Homo sapiens hypothetical protein FLJ10103 (FLJ10103)	1.5
Transcription factor BTEB2, Kruppel-like factor 5 (intestinal)	1.5
Kruppel-like factor (zinc finger binding protein)	1.5
Phosphoinositide-3-kinase	1.5
Adducin 3	1.5
PAPSS2 (sulfonation cascade)**	1.4
RIP140**	1.4
Karyopherin (importin) beta	1.4

Kruppel-like zinc finger protein	1.4
Androgen receptor	1.4
Solute carrier family 1	1.4
CACCC box binding protein/zinc finger protein 148	1.4
Kelch motif containing protein	1.4
Nuclear receptor interacting protein 1	1.4
Retinoblastoma-binding protein 6 (RBBP6)	1.4
Solute carrier family 1	1.3
CYP2A3**	1.3
Beta-glucoronidase**	1.3
gluccocorticoid receptor alpha 2**	1.3
PPAR binding protein/thyroid hormone receptor interactor 2**	1.3
CYP2A6**	1.3
putative transmembrane protein (NMA)**	1.3
Huntingtin interacting protein**	1.3
IGFBP5	1.3
SR-protein, transportin SR	1.3
SKAPP55 homolog	1.3
Methylmalonate-semialdehyde dehydrogenase	1.3
DEADH box binding protein	1.3
Human calmodulin mRNA	1.3
C-terminal binding protein 1	1.3
INSIG1 (insulin induced gene)**	1.3
ABC1 (ATP binding cassette protein)**	1.3
***Related to SXR & metabolism.*	
*Genes shown in **bold** were validated by QRT-PCR.*	

Table 4. Genes down-regulated in U133 Array in MCF-7 cells treated with rifampicin.

Gene description	***fold decrease***
Natual killer cell group 2-F (NKG2-F) mRNA	-2.5
Homo sapiens hypothetical protein FLJ20060	-1.9
Nuclear pore complex interacting protein	-1.7
EGF-containing fibulin-like extracellular matrix protein 1	-1.7
Human cytochrome b5 mRNA	-1.4
Cluster Incl. C18318, Homo sapiens cDNA	-1.3
Homo sapiens CDC14 homolog A (CDC14A)	-1.3
Similar to RIKEN cDNA C330013D18 gene	-1.3
Consensus includes gb:AW592266,v-myb oncogene homolog-like	-1.3
F-box only protein 5 (FBXO5)	-1.3
S-adenosylmethionine decarboxylase 1 (AMD1)	-1.2
Chromodomain helicase DNA binding protein 4 (CHD4)	-1.2

Table 5. Genes up-regulated in U133 Array in MDAMB231 cells treated with rifampicin.

Gene description	***fold increase***
Similar to rat myomegalin	5.3
Consensus includes gb:AK001970.1, Human alpha 1,2-mannosidase IB	3.7
gb:NM_018268.1 /DEF=Homo sapiens hypothetical protein	3.5
Homo sapiens paired box gene 4 (PAX4)	3.0
Fibroblast growth factor 7 (keratinocyte growth factor) (FGF7)	3.0
Homo sapiens dentin phosphoryn mRNA	2.8
Consensus includes gb:AK024615.1	2.8
Consensus includes gb:AI003579, solute carrier family 6	2.5
Homo sapiens neuregulin 1 (NRG1), transcript variant ndf43	2.5
Consensus includes gb:AI827820, methyl-CpG binding domain protein 2	2.1
Homo sapiens protein tyrosine phosphatase, non-receptor type 2 (PTPN2)	2.0
Consensus includes gb:AW189518, piwi (Drosophila)-like 1	2.0
Consensus includes gb:AI979276, Weakly similar to KIAA1276 protein	1.9
Homo sapiens G protein-coupled receptor 21 (GPR21)	1.9
Rho GDP dissociation inhibitor alpha	1.7
Homo sapiens G protein-coupled receptor 58 (GPR58)	1.7
Consensus includes gb:AW024335, CD27-binding (Siva) protein	1.7
Consensus includes gb:AW970584	1.7
Homo sapiens hormonally upregulated neu tumor-associated kinase (HUNK)	1.7
Human rho GDI mRNA	1.6
Homo sapiens hypothetical protein PRO1942 (PRO1942)	1.6
gb:BC004473.1 /DEF=Homo sapiens, clone MGC:10442	1.6
Homo sapiens zinc finger protein 237 (ZNF237)	1.6
Consensus includes gb:AL359578.1	1.6
Consensus includes gb:BE561596, metastasis associated 1	1.6
Human 68 kDa type I phosphatidylinositol-4-phosphate 5-kinase alpha mRNA	1.5
Disintegrin-like metalloprotease with thrombospondin type 1 motif, 3	1.5
Consensus includes gb:BF062364, olfactory receptor, family 7E24 pseudogene	1.5
Human N-methyl-D-aspartate receptor subunit NR3 (hNR3) mRNA	1.5
Homo sapiens prolactin (PRL)	1.5
Consensus includes gb:AB032967.1	1.5
Homo sapiens mannan-binding lectin serine protease 2 (MASP2)	1.5
H.sapiens mRNA for hcgVIII protein	1.5
cyclin A2 (CCNA2)	1.5
human chromosome 19 cosmid R31180	1.5
Consensus includes gb:AI859865, minichromosome maintenance deficient	1.5
Enoyl-Coenzyme A, hydratase3-hydroxyacyl Coenzyme A dehydrogenase	1.5
Homo sapiens toll-like receptor 4 (TLR4)	1.5
Consensus includes gb:AA699583, ARP2 (yeast homolog)	1.4
Consensus includes gb:X04014, Homo sapiens DNA for HBV integration sites	1.4
Consensus includes gb:S72422,alpha-ketoglutarate dehydrogenase complex	1.4
Homo sapiens insulin induced gene 1 (INSIG1)**	1.4
Homo sapiens inhibin, beta C (INHBC)	1.4
Consensus includes gb:AF131790, cortactin SH3 domain-binding protein	1.4
calreticulin	1.4

Homo sapiens guanine nucleotide binding protein (G protein), GNB2	1.4
gb:NM_018593, Homo sapiens hypothetical protein PRO0813 (PRO0813)	1.4
Homo sapiens latent transforming growth factor beta binding protein 3 (LTBP3)	1.4
Consensus includes gb:AI954041, F-box only protein 9	1.4
Consensus includes gb:AL571424, GCN5-like 2	1.4
Consensus includes gb:AI419423	1.4
Homo sapiens, GATA-binding protein 3	1.4
Homo sapiens vinexin beta (SH3-containing adaptor molecule-1) (SCAM-1)	1.4
Human zinc finger protein 45 (a Kruppel-associated box (KRAB) domain)	1.4
Homo sapiens trophinin associated protein (tastin) (TROAP)	1.4
Human (BAX delta) mRNA	1.4
Homo sapiens fibulin 1 (FBLN1), transcript variant D	1.4
H.sapiens mRNA for gonadotropin-releasing hormone receptor	1.3
2,3-bisphosphoglycerate mutase (BPGM)	1.3
Consensus includes gb:AK022679.1	1.3
Consensus includes gb:BE543527	1.3
Consensus includes gb:AJ302584,human 6M1-10*01, olfactory receptor	1.3
H.sapiens FASApo 1 mRNA for FAS soluble protein (clone FAS Exo4Del)	1.3
gb:NM_024303.1, Homo sapiens hypothetical protein MGC4161	1.3
Consensus includes gb:AK000918.1,similar to human VAMP-associated protein 33 kDa	1.3
Homo sapiens Fanconi anemia, complementation group C (FANCC)	1.3
Homo sapiens calmodulin 3 (phosphorylase kinase, delta) (CALM3)	1.3
C-terminal binding protein 1	1.3
ABC1 (ATP binding cassette protein)**	1.3
***Related to SXR & metabolism.*	
*Genes shown in **bold** were validated by QRT-PCR.*	

Table 6. Genes down-regulated in U133 Array in MDAMB231 cells treated with rifampicin.

Gene description	***fold decrease***
Consensus includes gb:AK023827, similar to mouse BANP (SMAR1) homolog	-13.9
Homo sapiens putative human HLA class II associated protein I (PHAP1)	-2.0
Human prostate carcinoma tumor antigen (pcta-1)	-1.9
Consensus includes gb:AI337584, nuclear mitotic apparatus protein 1	-1.6
Homo sapiens zinc finger homeobox 1B (ZFHX1B)	-1.6
Homo sapiens peroxisomal biogenesis factor 6 (PEX6)	-1.5
Consensus includes gb:AK021918, hypothetical protein FLJ11856	-1.4
Consensus includes gb:AL571362, calcium binding protein Cab45 precursor,	-1.4
Homo sapiens beta-1,3-glucuronyltransferase 3 (B3GAT3)	-1.4
Homo sapiens, clone MGC:12663, mRNA	-1.4
Homo sapiens G protein-coupled receptor 56 (GPR56)	-1.4
Homo sapiens hemochromatosis protein splice variant 562-878del (HFE)	-1.4
Homo sapiens hypothetical protein FLJ11196 (FLJ11196)	-1.4

Consensus includes gb:BE675435, core promoter element binding protein	-1.4
Consensus includes gb:AB033025, Homo sapiens mRNA for KIAA1199 protein	-1.4
Consensus includes gb:AK023637, Homo sapiens cDNA FLJ13575 fis	-1.3
Homo sapiens hypothetical protein FLJ20644 (FLJ20644)	-1.3
Human suppression of tumorigenicity 16 (melanoma differentiation) (ST16)	-1.3
Homo sapiens fetal Alzheimer antigen (FALZ)	-1.3
Consensus includes gb:AW163148,myrist'd alanine-rich protein kinase C substrate	-1.3
H.sapiens gene for plectin 1, intermediate filament binding protein, 500kD	-1.3
Consensus includes gb:AL157437, anchor attachment protein 1 (Gaa1p, yeast)	-1.3
Consensus includes gb:AW409974	-1.3
Consensus includes gb:BE675337, gelsolin (amyloidosis, Finnish type)	-1.3
Consensus includes gb:NM_002883, Ran GTPase activating protein 1 (RANGAP1)	-1.3
Homo sapiens BTB (POZ) domain containing 2 (BTBD2)	-1.3
Homo sapiens SEC24 (S. cerevisiae) related gene family, member C (SEC24C)	-1.3

Table 7. Genes commonly up-regulated by rifampicin treatment in both MCF-7 and MDAMB231 cell lines.

Gene description	**MCF7**	**231**
thrombospondin 1	1.7	1.5
2,3-bisphosphoglycerate mutase (BPGM)	1.8	1.3
gb:AW970584 (EST)	1.5	1.7
calreticulin	1.6	1.4
C-terminal binding protein 1	1.3	1.3
calmodulin	1.3	1.3
ABC1 (ATP binding cassette protein)	1.3	1.2
INSIG 1	1.3	1.4

Subsequent to completing the initial analysis of the two sets of microarray data, we began the process of validating the SXR target genes predicted from the microarrays using quantitative real-time RT-PCR (QRT-PCR). For this method, primer pairs were designed with annealing temperatures of about 60°C that would produce PCR products of between 80-200 basepairs for proper analysis. We screened each of the genes shown in bold in Tables 1-7 by QRT-PCR using the same RNA samples for MCF-7 and MDAMB231 used to probe the U133 array (the second microarray approach). The results showing the fold induction of each gene compared to untreated control for both the microarray and QRT-PCR are shown in Table 8. As a basis for comparison, we used the SXR target gene CYP3A4 as a positive control gene that should be up-regulated by treatment of SXR-expressing cells with RIF. As shown in Table 8, CYP3A4 was increased in the RIF-treated RNA samples from both cell lines, but at low levels. This low

increase in a bona-fide SXR target gene formed part of our basis for deciding that increases of at least 1.3 fold were to be considered significant in our results.

Table 8. Microarray validation using quantitative real-time RT-PCR (QRTPCR).

Primer	MCF7 RIF fold increase		MDAMB231 RIF fold increase		Function
	Microarray	QRTPCR	Microarray	QRTPCR	
Thrombospondin 1	1.7	1.0	1.5	1.3	Anti-angiogenic, induces apoptosis of cancer cells
Calreticulin	1.6	2.4	1.4	1.0	Calcium binding molecular chaperone, in ER
C-terminal binding protein 1	1.3	2.7	1.3	0.1	Ubiquitous co-repressor, apoptosis regulator
Calmodulin	1.2	0.6	1.3	1.1	Calcium binding, roles in growth, cell cycle and signal transduction
INSIG1	1.3	1.4	1.4	1.4	Cholesterol homeostasis
IGFBP4	3.4	1.8	no change	1.8	Insulin-like growth factor, control of cell proliferation
IGFBP5	1.3	1.9	no change	0.7	Insulin-like growth factor, control of cell proliferation
CACCC box binding protein	1.4	2.2	no change	1.1	Insulin-like growth factor receptor cascade target gene
IGF1R	1.3	7.8	no change	0.1	Insulin-like growth factor 1 receptor, control of apoptosis
GPR32	3.7	3.1	no change	ND	Novel G-protein coupled receptor homolog
Estrogen-responsive B Box protein	2.0	1.9	ND	0.2	Putative estrogen & tamoxifen-responsive transcription factor
TR3	6.3	1.8	ND	ND	Orphan receptor (Nurr77), apoptosis, cell cycle arrest
TGFBeta	4.6	1.3	ND	ND	Anti-apoptotic, role in development, cell proliferation, cell signalling
HES1	5.0	2.4	ND	ND	Human homolog of Drosophila Hairy, basic helix-loop-helix protein
Pilot	3.2	1.5	ND	ND	Putative transcription factor
B2 Bradykinin receptor	6.3	1.0	ND	ND	G-protein-coupled receptor, involved in inflammation
AhR	1.7	1.2	ND	ND	Aryl hydrocarbon receptor, suspected SXR target gene
CYP3A4	ND	1.5	ND	1.3	Cytochrome P-450, SXR target gene in liver & intestine

Unfortunately, the only gene predicted by microarray to be commonly up-regulated in both MCF-7 and MDAMB231 cells and subsequently validated by QRT-PCR was INSIG1, a known SXR target gene involved in cholesterol homeostasis. Of the other up-regulated genes tested, many that were predicted increased in microarray in one cell line or the other were validated by QRT-PCR. Amongst the MCF-7 genes chosen for validation, 14/17 genes predicted up by microarray were validated up by QRT-PCR. Fewer genes were checked using the MDAMB231 sample, and of those 4/9 predicted up by microarray were validated up by QRT-PCR.

Within the MCF-7 up-regulated genes, we noticed that several involved in the insulin-like growth factor signaling pathway were found by microarray. This signaling pathway has been implicated in control of cell proliferation and apoptosis, and therefore, we were interested in this

pathway as a mechanism to explain why the MCF-7 breast cancer cells stop proliferating in the presence of SXR activators. The insulin-like growth factor signaling pathway genes were found in the different microarray data sets and included Insulin-like growth factor binding proteins 4 and 5 (IGFBP4, IGFBP5), CACCC box binding protein (an IGFBP target gene) and insulin-like growth factor 1 receptor (IGF1R) (see Tables 1 & 3). Therefore, we checked these genes during our validation process. We found that each of them was up-regulated in the MCF-7 cells by both microarray and by QRT-PCR (Table 8). In the case of IGFBP5, a recent publication has shown that increased expression of IGFBP5 inhibited the growth of human breast cancer cell lines and resulted in both induction of apoptosis and a G2/M cell cycle arrest (Butt et al., 2003). We plan to follow up these initial findings with further analysis of the IGFBPs and other genes in this family using QRT-PCR. We will also closely examine the promoter regions of the insulin-like growth factor family members we have already found for evidence of SXR-dependent DNA regulatory elements.

Also among the up-regulated genes found by microarray in MCF-7 cells were several putative SXR target genes previously reported by another group performing microarray analysis to search for SXR target genes (Rosenfeld et al., 2003). This group used a custom made mouse microarray instead of a commercially available array such as Affymetrix. Table 9 shows a summary of the putative SXR target genes found by Rosenfeld et al. (2003) that were also found in our screen using MCF-7 cells. The fact that in two independent array experiments 7 commonly up-regulated genes were found gives us confidence that our array experiments were valid. It also shows that SXR-dependent transcriptional activation by RIF treatment occurred as expected in MCF-7 cells.

Table 9. SXR-target genes commonly up-regulated in our microarray experiments in MCF-7 cells and in Rosenfeld et al. (2003).

gene name	**function**
ABC1	ATP binding cassette drug and nutrient transporter
AhR	aryl hydrocarbon receptor
Beta-glucoronidase	microsomal glucoronidase, involved in natural substrate metabolism
Huntingtin interacting protein	interacts with Huntingtin, a protein involved in Huntington's disease
INSIG1	cholesterol homeostasis
PAPSS2	sulfonation cascade
putative transmembrane protein	similar to membrane-bound O-acyl transferase family (MBOAT)

Testing of putative SXR-responsive promoters and analysis of target gene regulation (months 18-36).

Treating the breast cancer cell lines MCF-7 or MDAMB231 with SXR activators leads to a decrease in proliferation of those cells. While we are continuing to analyze putative SXR target genes from our microarray experiments that could be responsible for the decreased proliferation we observe, we have performed some preliminary experiments to examine the mechanism behind the decreased proliferation. This could help us refine our search for the direct SXR targets found in the microarray that are actually responsible for the decreased proliferation by focusing our efforts on genes involved in certain pathways.

The decreased proliferation seen in the breast cancer cells when treated with SXR activators could be due to apoptosis, necrosis or cell-cycle arrest. To sort out which of these possibilities is responsible for the effect we see, we have first focused on differentiating between apoptosis and necrosis. For this purpose, several commercially available means of detecting apoptosis and necrosis are available. We have used the Cell Death Detection Elisa Plus (Roche) and the EnzChek Caspase-3 Assay Kit (Molecular Probes) to distinguish between the first two possibilities.

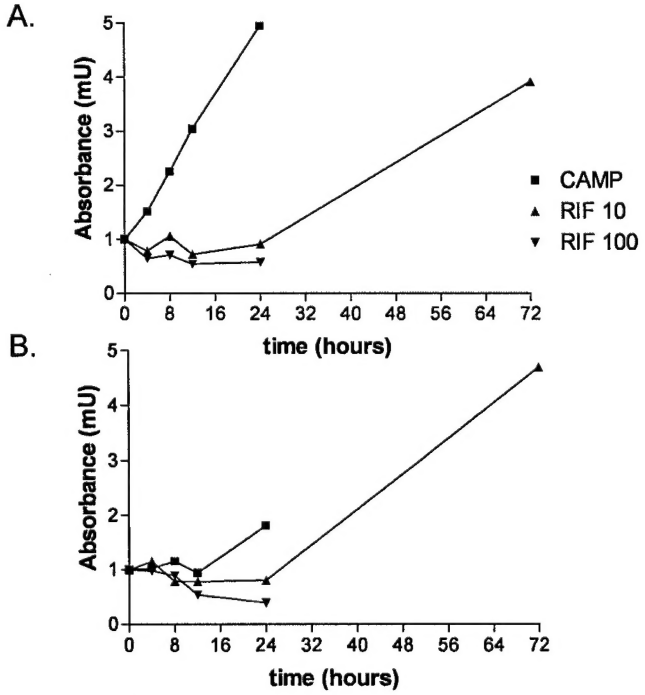

Figure 2. Cell Death Detection Elisa. A) DNA fragmentation in cell lysates from cells treated with 10 uM camptothecin (CAMP) or 10uM rifampicin (RIF 10) or 100 uM rifampicin (RIF 100). Absorbance units indicate apoptosis. B) DNA fragmentation in culture supernatants from cells treated as described in A). Absorbance units indicate necrosis.

The Cell Death Detection Elisa Plus can detect apoptosis and necrosis in a single experiment. We treated MCF-7 cells with either 10 μM or 100 μM of the SXR activator RIF for up to 24 hours. We also treated some cells with 10 μM rifampicin for a 72 hour timepoint. As a positive control for inducing apoptosis, we treated cells with 10 μM camptothecin (CAMP). In this experiment, the cell lysates and culture supernatants of treated cells are each measured for DNA fragmentation by Elisa. The cell lysates in which DNA

fragmentation has occurred contain the intact, apoptotic cells. The culture supernatants that demonstrate DNA fragmentation contain the previously lysed, necrotic cells. It is already known that after very long times of apoptosis, necrosis will become evident as well. As shown in Figure 2A, CAMP was able to induce apoptosis starting at 4 hours in the cell lysates of MCF-7 cells and increased apoptosis was seen at further times up to 24 hours. For either 10 μM or 100 μM RIF, no induction of apoptosis was seen by 24 hours, but we did see a strong induction using 10 μM RIF by 72 hours. Figure 2B shows the relative amount of necrosis as measured from the culture supernatants from the same treated cells. No necrosis is seen until longer times of CAMP or 10 μM RIF treatment (24 and 72 hours, respectively).

Treating the MCF-7 cells with 10 μM RIF induced apoptosis. Therefore, we wanted to see if other SXR activators were also able to induce apoptosis, and to differentiate whether or not caspase-dependent or caspase-independent apoptosis was occurring. Caspases have been shown to be crucial mediators of the complex biochemical events associated with apoptosis. For this purpose, we treated MCF-7 cells with 10 μM RIF or 10 μM clotrimazole (CLOT) for up to 72 hours. At different times after treatment, cells were trypsinized, frozen, and then lysed and assayed for caspase-3 activity using the EnzChek Caspase-3 Assay Kit. In this kit, a non-fluorescent substrate for enzymatic cleavage by caspase-3 is added to cell lysates. If capsase-3 activity is present, enzymatic cleavage converts the non-fluorescent substrate to a highly fluorescent product. Relative fluorescence compared to untreated control cells indicates the presence of capsase-3 activity. As shown in Figure 3, we found that both SXR activators RIF and CLOT were able to induce caspase-3 activity. Therefore, our preliminary experiments indicate that SXR activators induce apoptosis in MCF-7 breast cancer cells via a capsase-dependent mechanism.

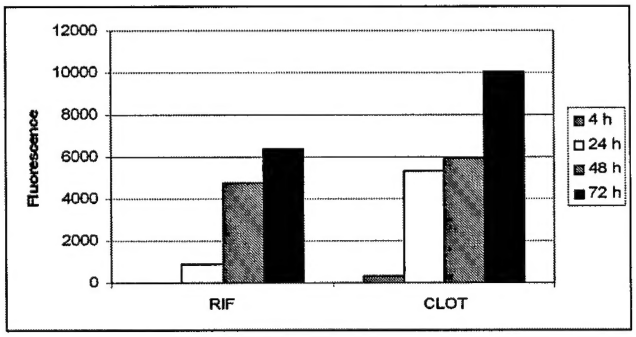

Figure 3. Caspase-3 activity assay. MCF-7 cells were treated with 10 uM rifampicin (RIF) or 10uM clotrimazole (CLOT) for 4, 24, 48 or 72 hours. Relative fluorescence indicates caspase-3 activity compared to untreated control.

KEY RESEARCH ACCOMPLISHMENTS

- Microarray results showing putative SXR target genes in MCF-7 and MDAMB231 cells.
- Validation of several up-regulated SXR target genes. Possible involvement of the insulin-like growth factor family.
- Apoptosis and necrosis assays demonstrate that treatment of MCF-7 cells with the SXR activator rifampicin induces apoptosis in a caspase-dependent manner.

REPORTABLE OUTCOMES

Presentations:

October, 2003 - Saitama University Medical School, Saitama, Japan
October, 2003 - National Institutes of Health Sciences, Tokyo, Japan
November, 2003 – Chao Comprehensive Cancer Center Symposium, Newport Beach, CA
December, 2003 – Department of Developmental and Cell Biology, UCI
January, 2004 - Department of Community and Environmental Medicine, UCI

CONCLUSIONS

One of the major challenges in breast cancer research is to develop new chemotherapeutic and chemopreventative agents, particularly for non-estrogen dependent breast cancers. SXR activators were able to slow the proliferation of ER+ and ER- breast cancer cell lines in culture, and a constitutively active form of SXR was also effective at slowing breast cancer cell growth. Expression of SXR mRNA in ductal carcinomas but not in normal tissue could mean that the presence or absence of SXR is an important prognostic marker for the success of breast cancer treatment. Upon treatment of breast cancer cell lines with SXR activators, many genes were found up and down-regulated by microarray. Several of these putative target genes in breast cancer cells were validated by quantitative real-time PCR and are currently under further study. Investigations into the manner in which SXR activators stop proliferation of breast cancer cells showed that at least in MCF-7 cells, treatment with the SXR activator rifampicin induces apoptosis in a caspase-dependent manner. Fully understanding the mechanisms through which SXR exerts its action by linking apoptosis with the SXR target genes found by microarray will provide opportunities for rational drug design and improvement of the efficacy of existing drugs that act through SXR.

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